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Efficient anaerobic production of succinate from glycerol in engineered *Escherichia coli* by using dual carbon sources and limiting oxygen supply in preceding aerobic culture

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HIGHLIGHTS

- Addition of acetate in the aerobic stage enhanced anaerobic succinate production.
- Aeration in the PCK induction stage affected the anaerobic metabolic capacities.
- 926.7 mM of succinate was produced in the anaerobic stage in a 1.5-L bioreactor.
- The yield of succinate in the anaerobic stage achieved 0.91 mol/mol.

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ABSTRACT

Glycerol is an important resource for production of value-added bioproducts due to its large availability from the biodiesel industry as a by-product. In this study, two metabolic regulation strategies were applied in the aerobic stage of a two-stage fermentation to achieve high metabolic capacities of the *pflB ldhA* double mutant *Escherichia coli* strain overexpressing phosphoenolpyruvate carboxykinase (PCK) in the subsequent anaerobic stage: use of acetate as a co-carbon source of glycerol and restriction of oxygen supply in the PCK induction period. The succinate concentration achieved 926.7 mM with a yield of 0.91 mol/mol during the anaerobic stage of fermentation in a 1.5-L reactor. qRT-PCR indicated that the two strategies enhanced transcription of genes related with glycerol metabolism and succinate production. Our results showed this metabolically engineered *E. coli* strain has a great potential in producing succinate using glycerol as carbon source.

1. Introduction

Non-food based renewable substrates are attractive feedstock to replace sugars to produce bio-based bulk chemicals, and is the new trend in bio-manufacturing. Biodiesel is one of the most promising alternate and renewable fuels, and its production generates about 10% (w/w) of glycerol as a byproduct (Yang et al., 2012). The fast growth of biodiesel production has generated large quantities of crude glycerol, leading to a drastic decrease in its price. Great attentions have been paid to development of new technologies to convert glycerol to value-added chemicals. Previous studies have shown that glycerol could be biologically converted to higher value products, such as 1,3-propanediol (Zhong et al., 2014), 3-hydroxypropionic acid (Huang et al., 2012), ethanol (Nikel et al., 2010), succinate (Choi et al., 2016), free fatty acids (Wu et al., 2014), and etc.

Succinate was evaluated by the U.S. Department of Energy as one of the top 12 building block chemicals produced from biomass (Werpy and Petersen, 2004). It is widely used in production of foods, pharmaceuticals, and biodegradable plastics (Zeikus et al., 1999). Traditionally, succinate is produced from petroleum-derived maleic anhydride, which, however, is unrenewable. A green bioprocess is urgently required to produce succinate from renewable resources. Many previous studies focused on converting sugars to succinate by bacteria, including *Actinobacillus succinogenes* (Mckinlay et al., 2005; Zhao et al., 2016), *Anaerobiospirillum succiniciproducens* (Lee et al., 2008), *Mannheimia succiniciproducens* (Choi et al., 2016), and metabolically engineered *Escherichia coli* (Vemuri et al., 2002). In this study, glycerol was chosen as the substrate for succinate production. Biosynthesis of succinate, a C_4-dicarboxylic acid, from glycerol needs fixation of CO_2, a major

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greenhouse gas which leads to the global warming. Succinate is synthesized aerobically as an intermediate of TCA cycle; it also can be synthesized from PEP through PCK or PPC enzymes under anaerobic fermentation conditions. The processes of succinate production could be combined with ethanol fermentation to reduce the emission of CO_2 as shown in our previous study (Wu et al., 2012). Theoretically, through anaerobic fermentation of glycerol, production of 1 mol succinate consumes 1 mol CO_2 (Fig. S1), indicating succinate production from glycerol can be an effective process for CO_2 fixation. In addition, the process to convert glycerol to succinate is redox balanced, and thus is superior to that using glucose. Therefore, it has great potential to improve the microbial succinate biosynthesis from glycerol.

E. coli does not naturally accumulate large amount of succinate in fermentation. To improve succinate production in *E. coli*, competitive pathways were disrupted, such as pyruvate:formate lyase (*pflB*), lactate dehydrogenase (*ldhA*) (Vemuri et al., 2002), alcohol dehydrogenase (*adhE*) (Sanchez et al., 2005a), and phosphate acetyltransferase-acetate kinase (Sanchez et al., 2005b). Another strategy to improve succinate production is to overexpress genes directly involved in the succinate synthesis pathway, including those coding for native or exogenous CO_2-fixation enzymes: phosphoenolpyruvate (PEP) carboxykinase (PCK) (Liu et al., 2012), PEP carboxylase (PPC) (Millard et al., 1996), pyruvate carboxylase (PYC) (Lin et al., 2004), and malic enzyme (ME) (Stols et al., 1997). Simplified metabolic pathways of succinate production form glycerol are shown in Fig. S1. *E. coli* can hardly grow under anaerobic conditions using glycerol as the carbon source without existence of exogenous electron acceptor (Lin, 1976). Recently, researchers reported the consumption of glycerol under particular anaerobic conditions (Dharmadi et al., 2006), but the glycerol consumption rate remained low. In order to increase production of succinate from glycerol, a series of genes, including *atpE*, *fdoH* and so on, were knocked out individually based on the results of simulation, and the fermentation results showed that disruption of these genes had positive effects on succinate produced from glycerol (Mienda et al., 2016a,b). The highest succinate concentration of these engineered strains increased to about 2 g/L after 7 days of fermentation. An engineered *E. coli* was constructed by interrupting *pflB* and *ptsI* together with mutation on the promoter of *pck* to increase its expression. As a result, 102 mM of succinate was accumulated in 144 h (Zhang et al., 2010). PYC from *Lactococcus lactis* was also overexpressed to convert glycerol to succinate under microaerobic condition by engineered *E. coli* strain, whose pathways of byproducts were blocked. The concentration of succinate reached to 118 mM in this case (Blankschien et al., 2010). Aerobic production of succinate from glycerol was also examined by using the engineered strain *E. coli* E2-*Δsdh-ppc-sucAB* (Li et al., 2013). Although the final concentration and productivity of succinate were higher than those obtained under anaerobic and micro-aerobic conditions, the succinate yield dropped dramatically. Previously, two-stage fermentation was used to enhance succinate production from sugars (Vemuri et al., 2002; Wu et al., 2007). We have constructed an *E. coli* strain MLB/pTrc99A-*pck* by deleting *pflB* and *ldhA* and overexpressing *pck*. This strain produced 118.1 and 360.2 mM of succinate in flasks and a 1.5-L bioreactor, respectively, in two-stage fermentation (Li et al., 2016a). Supplementary Table S1 summarizes the fermentation of *E. coli* to produce succinate with glycerol as the carbon source by various investigators.

Gene expression can be regulated by the carbon source under aerobic conditions in *E. coli* (Kao et al., 2005). When *E. coli* uses acetate as the carbon source for growth, the enzymes benefitting succinate production are up-regulated, including those in glyoxylate shunt, reductive TCA cycle, and gluconeogenesis (Oh et al., 2002; Wu et al., 2007). Meanwhile, *E. coli* has three pathways involved in the respiratory (aerobic respiratory, GlpK-GlpD; anaerobic respiratory, GlpK-GlpABC) and fermentative utilization of glycerol (fermentative route, GldA-DhaKLM) (Fig. S1) (Durnin et al., 2009). During the induction stage of PCK, changing the level of aeration, which affect the dissolved oxygen level of the culture, also can affect the gene expression of glycerol consumption and metabolism.

In this study, the metabolic regulation of the engineered strain, which was affected by the different aerobic cultivation strategies of the two-stage fermentation, was investigated for further increasing its succinate production from glycerol in the subsequent anaerobic stage. Acetate was used as a co-substrate in the aerobic stage to facilitate the consumption of glycerol in the subsequent anaerobic stage. Furthermore, it was found that limiting aeration during the induction of PCK was also critical to the improvement of glycerol consumption and succinate production.

2. Materials and methods

2.1. Strain and media

E. coli strain MLB (Δ*ldhA*::FRT Δ*pflB*::FRT) over-expressing *pck* (MLB/pTrc99a-*pck*) (Li et al., 2016a) was used exclusively in this study. This strain was stored in 25% (w/w) glycerol at −20 °C.

Luria-Bertani broth (LB), which contained (per liter) tryptone 10 g, yeast extract 5 g, and sodium chloride 10 g, was used for primary inoculum preparation. Ampicillin (100 mg/L) was included when needed.

Salt medium (SM) that was based on M9 contained (per liter) $Na_2HPO_4{\cdot}12H_2O$ 15.12 g, KH_2PO_4 3.0 g, NaCl 0.5 g, $MgSO_4{\cdot}7H_2O$ 0.5 g, $CaCl_2$ 0.011 g, NH_4Cl 1.0 g, 1% (w/v) vitamin B1 0.2 mL, and trace elements solution 0.1 mL. The stock solution of trace elements contained the following (per liter) in 3 M HCl: $FeSO_4{\cdot}7H_2O$ 80 g, $AlCl_3{\cdot}6H_2O$ 10 g, $ZnSO_4{\cdot}7H_2O$ 2.0 g, $CuCl_2{\cdot}2H_2O$ 1.0 g, $NaMoO_4{\cdot}2H_2O$ 2.0 g, $MnSO_4{\cdot}H_2O$ 10 g, $CoCl_2$ 4.0 g, and H_3BO_4 0.5 g.

For the two-stage fermentation in shake flasks, media GSM, GASM and ASM, which were prepared by supplementing SM with 54.3 mM (5 g/L) of glycerol, 32.6 mM (3 g/L) of glycerol plus 24.4 mM (2 g/L) of sodium acetate, and 61.0 mM (5 g/L) of sodium acetate, respectively. The medium for anaerobic fermentation in flasks was SM supplemented with 163.0 mM (15 g/L) of glycerol and 20 g/L of basic magnesium carbonate but without NH_4Cl.

The medium of fermentation carried out in a 1.5-L bioreactor was SM containing 543.3 mM (50 g/L) glycerol and ammonium acetate [64.8 mM (5 g/L) or 129.7 mM (10 g/L)]. In addition, the concentrations of $Na_2HPO_4{\cdot}12H_2O$ and KH_2PO_4 were changed to 3.78 and 0.75 g/L, respectively. Ampicillin was included at 100 mg/L.

2.2. Culture conditions

For two-stage fermentation carried out in flasks, the preculture was prepared by transferring 1 mL of the stock culture to 30 mL of LB in a 250 mL flask and aerobically incubated at 37 °C and 220 rpm for 8 h. 2-mL aliquots of the preculture were transferred to 500 mL flasks containing 100 mL GSM, GASM or ASM, in which the cells were incubated at 37 °C and 220 rpm for 8 (GSM, GASM) or 10 h (ASM). Then, IPTG was added to a final concentration of 0.1 mM, and the cells were incubated for another 4 h. The cells were harvested by centrifugation at 4 °C and 6300×*g* for 5 min, and were resuspended in the anaerobic fermentation medium at a cell density around 15 (OD_{600}). 20-mL aliquots of the cells suspension were dispensed to 50 mL schott bottles, and the headspace was filled with CO_2 to start the anaerobic culture at 37 °C and 220 rpm for 72 h. All the experiments were performed in triplicates.